Choose the kit or vector that's right for you

You can put the power of *Pichia pastoris* into your laboratory with a complete *Pichia* Expression Kit or with individual *Pichia* expression vectors. Three *Pichia* Expression Kits are available, each of which includes vectors, *Pichia pastoris* strains, reagents for transformation, sequencing primers, media, and a comprehensive manual (table 3). In addition, the pPICZ, pGAPZ, and pPIC6 vectors are available separately (see insert for ordering information). Choose the kit or vector that best meets your research needs and take advantage of the proven strength of *Pichia pastoris*.

Table 3 – Pichia expression kit components

	EasySelect™ *Pichia* Kit	Multi-copy *Pichia* Kit	Original *Pichia* Expression Kit
Vectors	pPICZ A, B, & C (20 µg each) pPICZα A, B, & C (20 µg each)	pPIC9K (20 µg) pPIC3.5K (20 µg) pAO815 (20 µg)	pPIC9 (10 µg) pPIC3.5 (10 µg) pHIL-D2 (10 µg) pHIL-S1 (10 µg)
Strains	X-33 (Mut$^+$, His$^-$) GS115 (Mut$^+$) KM71 (Mut^S) GS115/pPICZ/*lacZ* (control)	GS115 (Mut$^+$) KM71 (Mut^S) GS115/B-gal (control) GS115/albumin (control)	GS115 (Mut$^+$) KM71 (Mut^S) GS115/β-gal (control) GS115/albumin (control)
Transformation Reagents	*Pichia* EasyComp™ Kit (120 transformations)	Spheroplast Module (50 transformations)	Spheroplast Module (50 transformations)
Sequencing Primers	5′ *AOX1* 3′ *AOX1* α-factor	5′ *AOX1* 3′ *AOX1* α-factor	5′*AOX1* 3′*AOX1* α-factor
Media and Supplements	YP base medium (2 pouches)* YP base agar (2 pouches)* Yeast Nitrogen Base (1 pouch)** Zeocin™ (250 mg)	YP base medium (2 pouches)* YP base agar medium (2 pouches)* Yeast Nitrogen Base medium (1 pouch)**	YP base medium (2 pouches)* YP base agar medium (2 pouches)* Yeast Nitrogen Base medium (1 pouch)**

* Each pouch contains reagents to prepare 1 liter of medium.
** Each pouch contains reagents to prepare 500 ml of 10X YNB.

The power of *Pichia* in your lab

Make the most out of your expression. Put the power of *Pichia pastoris* into your laboratory and experience the advantages of a high-producing, easy-to-use, proven expression system. See the Ordering Information insert enclosed, then call Invitrogen and order today.

References:

1. Cregg, J.M. *et al.* (1993) *Bio/Technology* **11:** 905-910.
2. Waterham, H.R. *et al.* (1997) *Gene* **186(1):** 37-44.

Important Licensing Information

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Powerful, versatile tools, continued

Multi-copy integration

Several *Pichia* expression vectors are available that allow you to increase the number of copies of your gene of interest in *Pichia pastoris*. This may lead to higher expression of your protein. The pPIC3.5K and pPIC9K vectors carry the kanamycin resistance gene to allow selection of transformants in which multiple copies of a *Pichia* expression vector have spontaneously integrated. Spontaneous multiple insertion events can be identified by their increased resistance to Geneticin®. pAO815 provides an alternative to screening for multiple insertion events. This vector allows you to clone multiple copies of your gene into a single vector. With pAO815, you can control the number of copies of your gene you want to express. The pPIC3.5K, pPIC9K, and pAO815 vectors all carry the following features:

- *AOX1* promoter for high-level inducible expression
- *HIS4* gene for identification of transformants
- 3′ *AOX1* gene for targeted integration into the *Pichia* host genome

In addition, the pPIC9K vector carries the α-factor secretion signal to target recombinant protein expression to the growth medium. For more information about the pPIC3.5K, pPIC9K, and pAO815 vectors, please visit our web site at www.invitrogen.com.

Optimize with *Pichia* strains

Selection and expression in *Pichia pastoris* is made easier with our large collection of strains. These strains allow you to optimize expression and recovery of your protein of interest. Table 2 provides information to help you choose the appropriate strain.

Table 2 – *Pichia pastoris* strains

Strain	Genotype	Application
GS115	*his4*	Selection of expression vectors containing *HIS4*
X-33	wild-type	Selection of Zeocin™-resistant expression vectors
KM71	*his4, aox1::ARG4, arg4*	Selection of expression vectors containing *HIS4* to generate strains with Mut^S phenotype
KM71H	*aox1::ARG4, arg4*	Selection of Zeocin™-resistant expression vectors to generate strains with Mut^S phenotype
SMD1168	*his4, pep4*	Selection of expression vectors containing *HIS4* to generate strains without protease A activity
SMD1168H	*pep4*	Selection of Zeocin™-resistant expression vectors to generate strains without protease A activity

Methods

Pichia Strains

Introduction

Pichia pastoris is similar to *Saccharomyces cerevisiae* as far as general growth conditions and handling. You should be familiar with basic microbiological and sterile techniques, basic molecular biology, and protein chemistry. Some general references to consult are *Guide to Yeast Genetics and Molecular Biology*, (Guthrie and Fink, 1991), *Current Protocols in Molecular Biology*, (Ausubel *et al.*, 1994), *Molecular Cloning: A Laboratory Manual*, (Sambrook *et al.*, 1989), *Protein Methods*, (Bollag and Edelstein, 1991), and *Guide to Protein Purification*, (Deutscher, 1990).

Genotype of *Pichia* Strain

The *Pichia* host strains GS115 and KM71 have a mutation in the histidinol dehydrogenase gene (*his4*) which prevents them from synthesizing histidine. All expression plasmids carry the *HIS4* gene that complements *his4* in the host, so transformants are selected for their ability to grow on histidine-deficient medium. Spontaneous reversion of GS115 and KM71 to His^+ prototrophy is less than 1 out of 10^8.

The parent strain of KM71 has a mutated argininosuccinate lyase gene (*arg4*) that prevents it from growing in the absence of arginine. The wild-type *ARG4* gene was used to disrupt *AOX1*, creating KM71, a Mut^S, Arg^+, His^- strain.

Both GS115 and KM71 will grow on complex medium such as YPD (also known as YEPD) and on minimal media supplemented with histidine. Until transformed, neither GS115 nor KM71 will grow on minimal medium alone as they are His^-.

Construction of KM71

The *ARG4* gene (~2 kb) was inserted into the cloned, wild-type *AOX1* gene between the *Bam*H I site (codons 15/16 of *AOX1*) and the *Sal* I site (codons 227/228 of *AOX1*). *ARG4* replaces codons 16 through 227 of *AOX1*. This construct was transformed into the parent strain of KM71 (*arg4 his4*) and Arg^+ transformants were isolated and analyzed for the Mut^S phenotype. Genetic analysis of Arg^+ transformants showed that the wild-type *AOX1* gene was replaced by the *aox1::ARG4* construct.

Important

The advantage of using KM71 is that you do not need to screen for the Mut phenotype on methanol minimal medium. All transformants will be Mut^S. Secondly, since the *AOX1* locus was not completed deleted, it is theoretically possible to replace *aox1::ARG4* with your construct by gene replacement. The phenotype of this strain would be His^+, Mut^S, Arg^-. The recombinant strain would require arginine in the medium to grow. Unfortunately, simple inclusion of arginine does not totally alleviate the effects of the *arg4* mutation as *arg4* strains do not grow well on minimal medium containing arginine. We do not recommend generating His^+ transformants in KM71 by replacing the *aox1::ARG4* construct.

continued on next page

Pichia Strains, continued

Control Expression Strains

GS115/His$^+$ Mut^S Albumin: This strain is a control for secreted expression and the Mut^S phenotype when screening *Pichia* transformants (page 36). The gene for serum albumin was cloned with its native secretion signal, then integrated into *Pichia* at the *AOX1* locus. This strain secretes albumin (67 kDa) into the medium at levels > 1 gram/liter.

GS115/His$^+$ Mut$^+$ β-galactosidase: This strain is a control for intracellular expression and the Mut$^+$ phenotype when screening *Pichia* transformants (page 36). The *lacZ* gene was integrated into *Pichia* at the *his4* locus. This strain expresses β-galactosidase (117 kDa) at levels that can be visualized by Coomassie-stained SDS-PAGE (see pages 45-46) or assayed using ONPG (see page 65-66).

Growth of *Pichia* Strains

The growth temperature of *Pichia pastoris* is 28-30°C for liquid cultures, plates, and slants. Growth above 32°C during induction can be detrimental to protein expression and can even lead to cell death. Other important facts:

- Doubling time of log phase Mut$^+$ or Mut^S *Pichia* in YPD is ~2 hours
- Mut$^+$ and Mut^S strains do not differ in growth rates unless grown on methanol
- Doubling time of log phase Mut$^+$ *Pichia* in methanol medium (MM) is 4-6 hours
- Doubling time of log phase Mut^S *Pichia* in MM is ~18 hours
- One OD_{600} = ~5 x 10^7 cells/ml

Note: Growth characteristics may vary depending on the recombinant strain.

Growth on Methanol

When plates or medium containing methanol are used as growth medium, it is advisable to add methanol every day to compensate for loss due to evaporation or consumption.

- For plates add 100 µl of 100% methanol to the lid of the inverted plate.
- For liquid medium add 100% methanol to a final concentration of 0.5%.

Some researchers have had success adding methanol to 1% every day for Mut^S strains and up to 3% for Mut$^+$ without any negative effect to their liquid culture.

Make frozen stocks for long-term storage of all three *Pichia* strains included in this kit (see below).

Storage of *Pichia* Strains

To store cells for weeks to months, use YPD medium or YPD agar slants (see page 55).

- Streak for single colonies of GS115, KM71, or a His$^+$ transformant on YPD.
- Transfer one colony to a YPD stab and grow for 2 days at 30°C.
- The cells can be stored on YPD for several weeks at +4°C.

To store cells for months to years, store frozen at -80°C.

- Culture a single colony of GS115, KM71, or a His$^+$ transformant overnight in YPD.
- Harvest the cells and suspend in YPD containing 15% glycerol at a final OD_{600} of 50-100 (approximately 2.5-5.0 x 10^9 cells/ml).
- Cells are frozen in liquid nitrogen or a dry ice/ethanol bath and then stored at -80°C.

After long-term storage at +4°C or -80°C, we recommend checking the His$^+$ transformants for correct genotype and viability. Streak on MM, MD or MGY plates before using again.

BSSL variants. The prepared DNA was digested with BamHI, fractionated on agarose gels and transferred to membranes for hybridization. The probe used was ^{32}P-labelled BSSL cDNA. The hybridization results confirmed the presence of the recombinant genes and also that the vector copy number was approximately equal in the different cell lines (FIG. 2). The positions of the hybridizing fragments reflected the different lengths of the various BSSL sequences and were in agreement with the expected sizes. The positions were also similar to the bacteria derived DNA used in the transfection experiment, indicating that no major rearrangement of vector DNA had occurred in the cell lines (FIG. 2). The upper hybridization signals in the DNA sample representing variant A were probably due to partial digestion.

1.2.3. Expression of mRNA for full-length and mutated BSSL in mammalian cells

To analyze the expression of the different recombinant BSSL genes RNA was prepared from the isolated cell lines. Northern blot experiments and hybridization with ^{32}P-labelled BSSL cDNA showed that recombinant mRNA was detectable in all cell lines harboring a BSSL vector (FIG. 3). No hybridization was found in the control sample derived from a cell line containing an identical vector except for BSSL cDNA (FIG. 3).

The different lengths of the hybridizing mRNAs were in accordance with the modifications of the cDNAs. The steady state levels of recombinant BSSL mRNA variants in the different samples were about the same except for variant A (FIG. 3). The reason for the reduced accumulation of variant A mRNA is not known, but it was observed with two populations of cell lines as well as with isolated clones. The presence of equal amounts of RNA in the different samples was confirmed by hybridization to a murine β-actin probe (FIG. 3, lower panel).

1.2.4. Production of full-length and variants of BSSL in mammalian cells

Media from individual clones of the C127-cells, transfected with full-length BSSL and the different mutated forms, were collected and assayed for BSSL activity (FIG. 4). For the full-length molecule and variants N, B and C the activities in the clones with the highest expression ranged from 0.7 to 2.3 μmol fatty acid released×min^{-1}×ml of medium^{-1}. With a specific activity comparable to that of the native milk BSSL this would correspond to expression levels of 7–23 μg×ml medium^{-1}. For variant A all the analyzed clones had activities below 0.05 μmol fatty acid released× min^{-1} and ml of medium^{-1}. Concentration on Blue-Sepharose and lyophilization of the done showing the highest activity revealed that an active enzyme indeed was expressed, albeit at very low levels. The possibility that the low activity obtained with variant A in part could be explained by a considerably lower specific activity could not be ruled out.

Western blots from clones of the different transfection experiments are shown in FIG. 5A. The apparent M_r of the BSSL variants were as expected. It should be noted, however, that for full-length BSSL as well as for variants B and C a double band was obtained. Because all three have the single N-glycosylation site intact whereas variant N, which showed no double band, lacks that site, a likely explanation was that the double band resulted from differences in N-glycosylation. Therefore variant B was subjected to digestion with N-glycosidase F. As shown in FIG. 5B, only trace amounts of the upper band remained while the lower band increased in strength indicating that only part of the expressed variant was N-glycosylated.

One of the characteristics of BSSL is its specific activation by primary bile salts, e.g. cholate (Hernell, 1975). All the different recombinant forms of BSSL showed the same concentration dependency for cholate activation (FIG. 6). A maximal activity was obtained at about 10 mM in the assay system used. When cholate was exchanged for deoxycholate (a secondary bile salt) no such activation occurred. Thus, the recombinant full-length as well as the different variants showed the same specificity regarding bile salt activation.

1.2.5. Expression and biochemical characterization of full-length BSSL in *E.coli*

Two *E.coli* strains JM109(DE3) and BL21(DE3)pLysS (Studier et al., 1986) were transformed with the expression vector pGEMEX/BSSL containing the human BSSL cDNA under control of the T7 promoter. Transformants from both strains were identified, cultured and induced with IPTG for about 90 min (Studier et al., 1986). Analysis of total mRNA by Northern blot using the BSSL cDNA as a ^{32}P-labeled probe demonstrated that expression was efficiently induced in both strains and that the transcription was tightly regulated (FIG. 7A). The apparent size of the recombinant BSSL mRNA, appoximately 2.4 kb, is in agreement with the expected length. SDS-PAGE separation of protein samples and immunodetection with anti-BSSL antibodies showed that full-length BSSL was efficiently produced in *E.coli* (FIG. 7B). More of the protein was secreted to the periplasm in the BL21(DE3)pLysS strain than in JM109(DE3) (FIG. 7B).

IPTG-induced *E.coli* cultures contained active soluble BSSL corresponding to 0.5–4 μg of BSSL protein/ml culture. Western blotting showed that between 20 and 60% of the reactive material was in the insoluble pellet. Uninduced bacteria did not contain any significant BSSL activity.

The lipase activity from cultured bacteria showed the same bile salt dependence as native milk BSSL.

2. PURIFICATION AND CHARACTERIZATION OF RECOMBINANT FULL-LENGTH AND MUTATED FORMS OF BILE SALT-STIMUATED LIPASE

2.1. EXPERIMENTAL PROCEDURES

2.1.1. Enzymes and enzyme variants

Recombinant full-length BSSL and BSSL variants B, C and N were constructed and expressed as previously described. Compared to the native enzyme Variant B (SEQ ID NO: 5) lacks all 16 unique, O-glycosylated, proline-rich, C-terminal repeats (aa 536–711) but with the most C-terminal fragment (aa 712–722) fused to glutamine-535. Variant C (SEQ ID NO: 6) contains the same C-terminal fragment and two repeats of 11 residues between glutamine-535 and lysine-712. In variant N (non-N-glycosylated variant, SEQ ID NO: 7) the asparagine-187 responsible for the only N-linked sugar was exchanged for a glutamine residue.

Native BSSL was purified from human milk as described (Bläckberg & Hernell, 1981).

2.1.2. Enzyme assay

Lipase activity was assayed as described (Bläckberg & Hernell, 1981) using triolein emulsified in gum arabic as substrate. Sodium cholate (10 mM) was used as activating bile salt. Different modifications of the assay are given in legends to figures.

2.1.3. Preparation of immunosorbent

Purified milk BSSL (5 mg) was coupled to Sepharose using CNBr as described by the manufacturer. 40 ml of a polyclonal antiserum raised in rabbit against purified milk BSSL was passed over the column. Specific antibodies were eluted with 0.1 M glycine-HCl, pH 2.5. The pH was immediately adjusted to approx 8 with solid Tris. After desalting